IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Amanda Johanne Kiliaan et al.

Serial No.: 09/899,922

Filed: July 9, 2001, Continuation-in-part of May 2, 2000 (09/566,386)

Title: METHOD AND PREPARATION FOR PREVENTING AND/OR TREATING OF VASCULAR DISORDERS AND SECONDARY DISORDERS ASSOCIATED THEREWITH

Conf: 5229
Group: 1651
Examiner: Ruth A. Davis

October 25, 2004

DECLARATION UNDER RULE 132

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Ladislaus Maria Broersen, residing at Kneppelhoutstraat 27, Utrecht, The Netherlands, do hereby declare that:

1. I am a citizen of the Netherlands,

2. My educational and technical background in the field of neuropsychopharmacology is as follows:

a) I am a M.Sc. graduate from the Department of Psychopharmacology, University of Utrecht, Utrecht, the Netherlands, specialization in neuropsychopharmacology;

b) From January 1990 till January 1991, I was employed at the Faculty of Pharmacy,

University of Utrecht, Utrecht, The Netherlands; from April 1991 till June 1995, I was employed at the KNAW, Netherlands Institute for Brain Research, Amsterdam, The Netherlands; from November 1995 till August 1996, I was employed at the Department of behavioural Biology, ETH Zürich, Schwerzenbach, Switzerland; from September 1996 till July 1999, I was employed at NWO-GPD, Netherlands Institute for Brain Research, Amsterdam, The Netherlands;

c) I have been employed by Nutricia N.V. since January 1, 2000, presently as a senior scientist dealing with research and the process design of clinical nutrition.

3. I have read Kiliaan et al. US application 09/899,922 filed July 9, 2001;

4. I make this declaration in support of the present application, and to provide evidence demonstrating that one of ordinary skill in the art would not find the presently-claimed invention obvious in view of several publications cited in the Office Action mailed on May 4, 2004.

I believe that the enclosed data (Annex I and Annex II) show that the claimed combination is unexpectedly effective for the treatment of depression and its related disorders. The data show that the claimed combination of fatty acids, phospholipids and methionine metabolism factors (supplement I of Annex II) is better than a diet of fatty acids (control diet of Annex I), and also better than a diet of vitamins and fatty acids supplemented with the ω-3 fatty acids DHA and EPA (supplement II of Annex II).

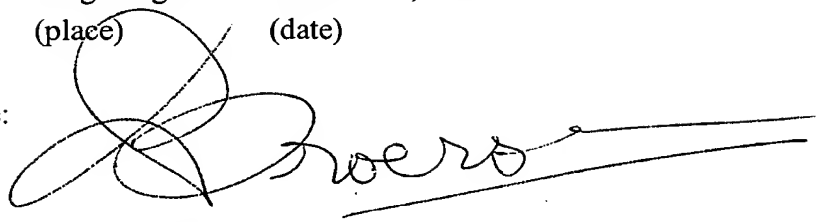
Moreover, Table I of Annex II shows that the supplement comprising the claimed combination provides a good antidepressant effect. This is further confirmed from the comparison of the graphs of Annex I and Annex II, wherein bulbectomized animals treated with the supplement according to Annex II show less hyperactivity, and thus reduced depression than in any other case.

Thus, the claimed method for treating unipolar depression comprising the administration of fatty acids, phospholipids and a methionine metabolism factor, let alone in the present ratios or in co-administration with citrate and/or vitamin D3, is not disclosed or suggested in any combination of literature cited by the Examiner.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing

thereon.

Dated: Wageningen (place) October 25, 2004 (date)

Signature: 

Ladislaus Maria Broersen

ANNEX I

SUPPLEMENTATION OF OMEGA-3 PUFAS DOES NOT AFFECT BEHAVIOURAL CHANGES INDUCED BY THE OLFACTORY BULBECTOMY MODEL OF DEPRESSION

Introduction

The olfactory bulbectomized (OBX) rat has been proposed as an animal model of depression [Kelly et al (1997) The olfactory bulbectomized rat as a model of depression: an update. *Pharmacol Ther* 74:299-316]. Following bilateral olfactory bulbectomy, a number of behavioural changes has been observed, including hyperactivity in the open field and deficits in memory in several maze procedures. Typically, behavioural changes induced by OBX are attenuated by chronic (but not acute) antidepressant treatment.

The most commonly employed behavioural indicator of antidepressant activity is attenuation of the OBX-related hyperactivity in the open field. In the present experiment, we investigated the effects of a dietary supplementation of the omega-3 polyunsaturated fatty acids (PUFAs) DHA and EPA on activity changes induced by the OBX model of depression.

Materials and methods

Animals

Twenty-four male Sprague Dawley rats (Harlan, The Netherlands) were 8 weeks old at the start of the experiment and were housed in groups of 4 rats per cage. Rats were housed at controlled room temperature (21 ± 2 °C) and relative humidity of 60 ± 10% under reversed 12 h light – 12 h dark cycle conditions (lights on at 19:00). Tap water and food were freely available throughout the experiment. Starting one day after surgery, standard rodent food (Special Diet Services, Witham, Essex, UK) was replaced by either a control diet or a supplement diet (Table 1). All experiments were carried out with the approval of the Animal Ethics Committee of the Faculties of Pharmacy, Chemistry and Biology of Utrecht University.

Olfactory bulbectomy

After a 4-week acclimatization period, two rats per cage underwent surgery to produce bilateral olfactory bulbectomy and two rats per cage underwent sham surgery. Rats were

anaesthetised with Hypnorm (0.7 ml/kg, s.c.) and Dormicum (0.5 ml/kg, s.c.) and two burr holes (2 mm diameter) were drilled in the skull, at 8 mm anterior to bregma, on either side 2 mm from the midline of the frontal bone overlying the olfactory bulbs. The olfactory bulbs were first damaged with the drill and were then aspirated by means of a blunt hypodermic needle attached to a water pump. Prevention of blood loss from the burr holes was achieved by filling them with haemostatic sponge. Sham-operated rats were treated similarly, except that the olfactory bulbs were neither damaged nor removed. One sham-operated animal did not survive surgery.

Diet compositions

The control diet was based on the composition of a standard rodent food. For the supplement diet, part of the soy bean oil was replaced by omega-3 PUFA-rich fish oil. A detailed description of the compositions of the control diet and the supplement diet are provided in Table 1. Both diets were stored in sealed plastic bags at -20 °C until use.

Locomotor activity

Spontaneous locomotor activity was measured both one week before surgery and three weeks after surgery. To this end, rats were individually placed in the centre of a square open field arena (75 × 75 × 50 cm) made of dark grey plastic, and locomotor activity was monitored for 30 min using an automated video tracking system for behavioural registration and analysis (Ethovision, Noldus, Wageningen, The Netherlands).

Table 1: Compositions of the control diet and the supplement diet that were used in the present study.

	Component	Control g/100 g	Supplement g/100g
Oil	Soybean oil	5	2.84
	Marinol C45 .4%		2.16
Proteins	Acid Casein	20	20
	DL-Methionine	0.2	0.2
Carbohydrates	Corn starch	10	10
	Cerelose	52.95	52.95
	Cellulose	5	5
Minerals	CaHPO4*2H2O	1.3	1.3
	CaCO3	1	1
	KH2PO4	0.7	0.7
	KCl	0.7	0.7
	NaCl	0.3	0.3
	MgSO4*7H2O	0.4	0.4
	MgO	0.2	0.2
	Inositol	0.05	0.05
Vitamins	Vitamin mix	1.2	1.2
Minerals	Mineral premix	1	1
Total		100	100

Results

The effects of OBX on spontaneous locomotor activity in rats fed with either the control diet or the supplement diet, are shown in figure 1. Data obtained after surgery are expressed as percentage of control scores. A two-way univariate analysis of variance with the between-subject factors Surgery (Sham or OBX) and Diet (Control or Supplement) revealed a main effect of the factor Surgery [$F(1,19)=12.07$, $p<0.005$], indicating that OBX rats showed an increase in locomotor activity. However, there was no significant effect of Diet, nor a significant Surgery $\times$ Diet interaction [both $p>0.60$]. These data show that the consumption of the supplement diet for 3 weeks, does not attenuate the behavioural changes induced by the OBX model of depression, indicating that the supplement diet has little antidepressant potential.

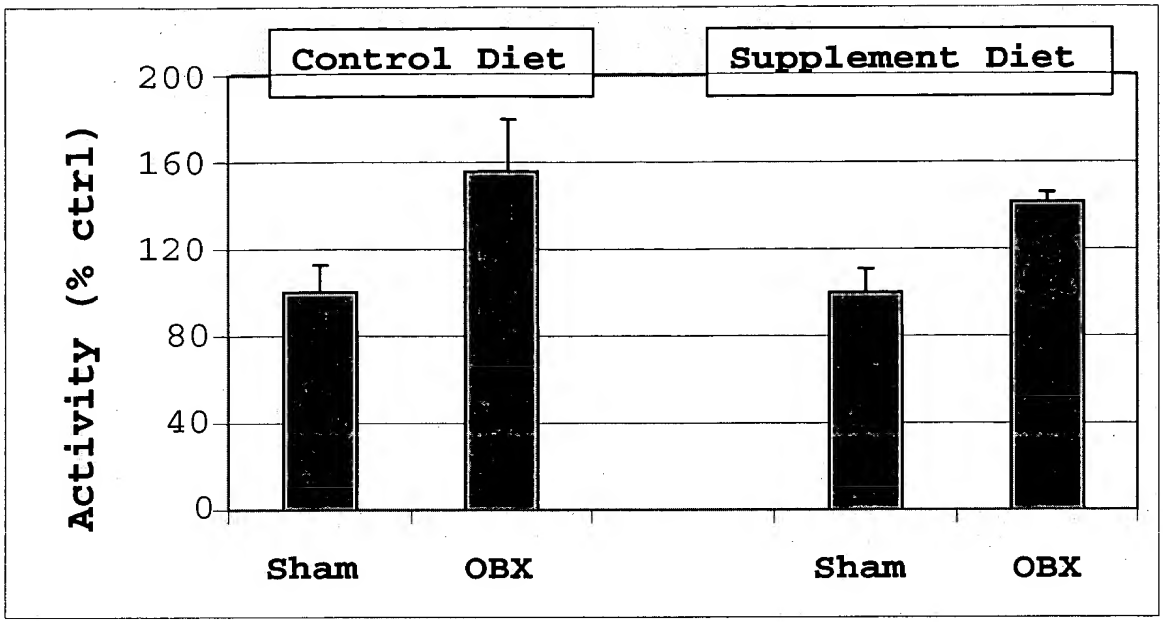


Figure 1: Effects of dietary omega-3 PUFA (DHA and EPA) supplementation on activity changes induced by olfactory bulbectomy (OBX), a rat model of depression. The increase in locomotor activity that is normally observed after OBX, is present in both diet groups, indicating that the PUFA-enrichment in the supplement diet by itself is insufficient to induce an antidepressant-like effect in the OBX model.

ANNEX II

THE EFFECTS OF A DIETARY SUPPLEMENT ON BEHAVIORAL CHANGES INDUCED BY THE OLFACTORY BULBECTOMY MODEL OF DEPRESSION

Introduction

The olfactory bulbectomized (OBX) rat has been proposed as an animal model of depression [Kelly et al (1997) The olfactory bulbectomized rat as a model of depression: an update. *Pharmacol Ther* 74:299-316]. Following bilateral olfactory bulbectomy, a number of behavioral changes has been observed, including hyperactivity in the open field and deficits in memory in several maze procedures. Typically, behavioral changes induced by OBX are attenuated by chronic (but not acute) antidepressant treatment.

The most commonly employed behavioral indicator of antidepressant activity is attenuation of the OBX-related hyperactivity in the open field. In the present experiment, we investigated the effects of a dietary supplement on activity changes induced by the OBX model of depression.

Materials and methods

Animals

Thirty-two male Sprague Dawley rats (Harlan, The Netherlands) were 8 weeks old at the start of the experiment and were housed in groups of 4 rats per cage. Rats were housed at controlled room temperature (21 ± 2 °C) and relative humidity of 60 ± 10% under reversed 12 h light – 12 h dark cycle conditions (lights on at 19:00). Tap water and food were freely available throughout the experiment. Starting one day after surgery, standard rodent food (Special Diet Services, Witham, Essex, UK) was replaced by either a control diet or a supplement diet (Table 1). All experiments were carried out with the approval of the Animal Ethics Committee of the Faculties of Pharmacy, Chemistry and Biology of Utrecht University.

Olfactory bulbectomy

After a 4-week acclimatization period, two rats per cage underwent surgery to produce bilateral olfactory bulbectomy and two rats per cage underwent sham surgery. Rats were anaesthetized with Hypnorm (0.7 ml/kg, s.c.) and Dormicum (0.5 ml/kg, s.c.) and two burr holes (2 mm diameter) were drilled in the skull, at 8 mm anterior to bregma, on either side 2 mm from the midline of the frontal bone overlying the olfactory bulbs. The

olfactory bulbs were first damaged with the drill and were then aspirated by means of a blunt hypodermic needle attached to a water pump. Prevention of blood loss from the burr holes was achieved by filling them with haemostatic sponge. Sham-operated rats were treated similarly, except that the olfactory bulbs were neither damaged nor removed. Two OBX rats did not survive surgery.

Diet compositions

The control diet was based on the composition of a standard rodent food. To the supplement diet, several food components were added, including DHA, EPA, phosphatidylserine, folic acid, Se, Cu, Mg, Zn, and vitamins B6, B12, D3 and E. A detailed description of the compositions of the control diet and the supplement diet are provided in Table 1. Both diets were stored in sealed plastic bags at -20 °C until use. The average intake per animal is of about 20g per day of food.

Locomotor activity

Spontaneous locomotor activity was measured both one week before surgery and three weeks after surgery. To this end, rats were individually placed in the centre of a square open field arena (75 × 75 × 50 cm) made of dark grey plastic, and locomotor activity was monitored for 30 min using an automated video tracking system for behavioral registration and analysis (Ethovision, Noldus, Wageningen, The Netherlands).

Table 1: Compositions of the control diet and the supplement diet that were used in the present study.

	Component	Control g/100 g	Supplement g/100g
Oil*	Soybean oil	5	2.84
	Marinol C45 .4%		2.16
Proteins	Acid Casein	20	20
	DL-Methionine	0.2	0.2
Carbohydrates	Corn starch	10	10
	Cerelose	52.95	50.70
	Cellulose	5	5
Minerals	$CaHPO_4*2H_2O$	1.3	1.3
	$CaCO_3$	1	1
	KH_2PO_4	0.7	0.7
	KCl	0.7	0.7
	NaCl	0.3	0.3
	$MgSO_4*7H_2O$	0.4	0.8
	MgO	0.2	0.4
	Inositol	0.05	0.05
Vitamins	Vitamin mix	1.2	1.2
Minerals	Mineral premix	1	1
Extras	Phosphatidyl serine** (Leci-PS_20P)		1.11
	Vitamine B6		0.0054
	Vitamine B12		0.02
	Foliumzuur		0.0004
	Vitamine E		0.5
	Vitamine D3		0.0004
	Na_2SeO_3*5aq		6.66E-05
	$ZnSO_4*aq$		0.006587
	$CuSO_4*5aq$		0.003145
Total		100	100

*oil comprises both ω-3 fatty acids and ω-6 fatty acids

**phosphatidyl serine (PS) comes from Leci-PS_20P which is a lecithin comprising 18-24% PS, min. 15% phosphatidyl choline (PC), max.
18% phosphatidyl ethanolamine (PE) and max. 10% phosphatidyl inositol (PI).

Results

The effects of OBX on spontaneous locomotor activity in rats fed with either the control diet or the supplement diet are shown in figure 1. Data obtained after surgery are expressed as percentage of preoperative scores. A two-way univariate analysis of variance with the between-subject factors Surgery (Sham or OBX) and Diet (Control or Supplement) revealed a main effect of the factor Surgery [$F(1,26)=6.51$, $p<0.02$], indicating that OBX rats showed an increase in locomotor activity. In addition, a Surgery $\times$ Diet interaction [$F(1,26)=4.78$, $p<0.05$] and post-hoc comparisons indicated that the increase in activity was present in the OBX rats fed with the control diet, but not in the OBX rats fed with the supplement diet, as compared to the respective Sham-operated groups. These data show that the consumption of the supplement diet for 3 weeks, attenuates the behavioral changes induced by the OBX model of depression, indicating that the supplement diet has antidepressant potential.

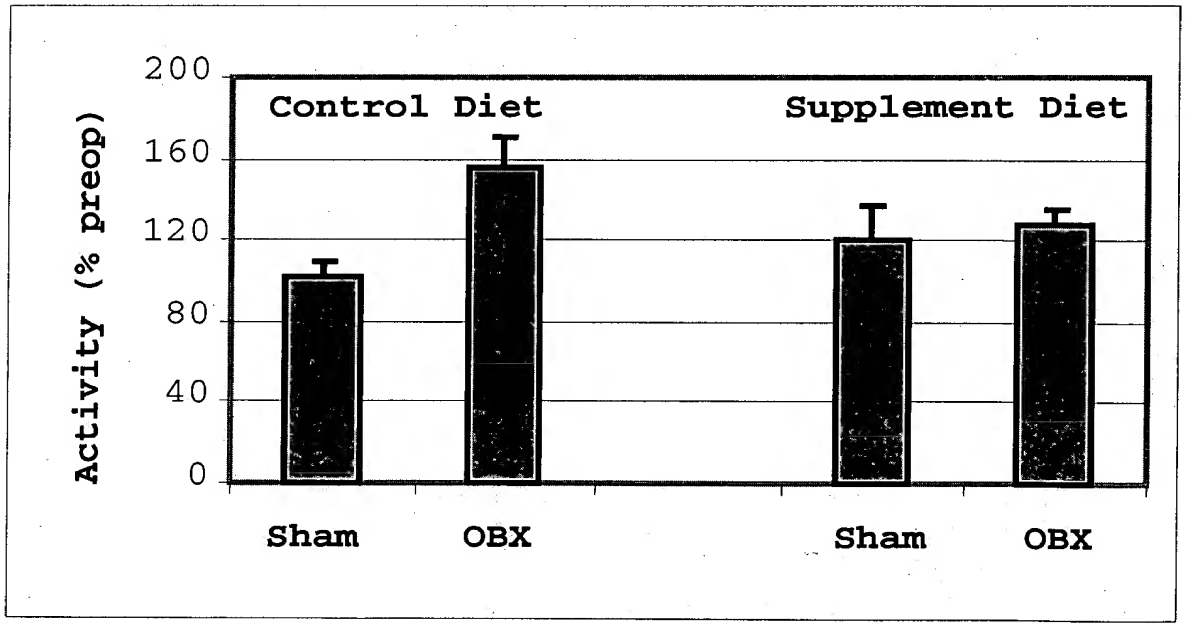


Figure 1: Effects of dietary supplement on activity changes induced by olfactory bulbectomy (OBX), a rat model of depression. The increase in locomotor activity that is normally observed after OBX, is present in the control diet groups, but not in the supplement diet groups. To the supplement diet, several food components were added, including DHA, EPA, phosphatidylserine, folic acid, Se, Cu, Mg, Zn, and vitamins B6, B12, D3 and E.